DuoSet® IC

Human/Mouse/Rat Active SHP-2

Catalog Number DYC2809

For the development of a phosphopeptide substrate assay to measure Src Homology region 2 (SH2) domain Phosphatase 2 (SHP-2) activity in cell lysates.

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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PRINCIPLE OF THE ASSAY

This DuoSet® IC activity assay contains the basic components required for the development of immunoprecipitation capture assays to measure the activity of human, mouse, and rat Src Homology region 2 (SH2) domain Phosphatase 2 (SHP-2) in cell lysates. SHP-2 is also known as PTP1D, SH-PTP2, PTP2C, PTPN11, and Syp. An anti-SHP-2 antibody conjugated to agarose immunoprecipitation (IP) beads is used to bind both active and inactive SHP-2. After washing away unbound material, a synthetic phosphopeptide substrate is added that is dephosphorylated by active SHP-2 to generate free phosphate and unphosphorylated peptide. The beads are pelleted by centrifugation and the supernatant is transferred to a microplate. The amount of free phosphate in the supernatant is determined by a sensitive dye-binding assay using malachite green and molybdic acid. By calculating the rate of phosphate release, the activity of SHP-2 is determined.

MATERIALS PROVIDED

Store the unopened kit at 2 - 8° C. Do not use past kit expiration date.

Description	Part #	Storage Conditions
Active SHP-2 Immunoprecipitation Beads	841914	2 - 8° C
Tyrosine Phosphatase Substrate I	841915	2 - 8° C
Phosphate Standard	892809	2 - 8° C
Malachite Green Reagent A	895855	2 - 8° C
Malachite Green Reagent B	895856	2 - 8° C

This Human/Mouse/Rat Active SHP-2 DuoSet IC Activity Assay contains sufficient materials to run approximately 50 IP assays (25 samples in duplicate) provided the following conditions are met:

- The reagents are prepared as described in this package insert.
- The assay is run as described in the General Assay Protocol on page 6.
- The recommended microplates, buffers, diluents, substrates, and solutions are used.

If additional reagents are required, a Malachite Green Phosphate Detection Kit (Catalog # DY996) and Tyrosine Phosphatase Substrate I (Catalog # ES006) are available from R&D Systems.

OTHER MATERIALS REQUIRED

- Aprotinin (Sigma # A6279)
- Leupeptin (Sigma # L8511)
- Pepstatin (Sigma # P4265)
- Phenylmethylsulfonylfluoride (PMSF) (Sigma # P7626)
- Sodium orthovanadate (Na_3VO_4) (Sigma # S6508)
- Dithiothreitol (DTT) (Sigma # D0623)
- NP-40 Alternative (EMD/Calbiochem # 492016)
- Deionized or distilled water
- Pipettes and pipette tips
- 1.5 mL microcentrifuge tubes
- 37° C incubator
- Plate reader capable of measurements at a wavelength of 620 nm
- Centrifuge capable of reaching 12,000 x g
- Rocking platform
- 96 well microplates [Costar EIA Plates (Catalog # 2592 or R&D Systems Catalog # DY990) are suggested]. For measurement of very low phosphatase activity, use 1/2 area microplates with a suitable plate reader [Costar EIA Plates (Catalog # 3690) are recommended]
- Plate sealers (R&D Systems, Catalog # DY992)
- Squirt bottle, manifold dispenser, or automated microplate washer

SOLUTIONS REQUIRED

TBS - 25 mM Tris, 150 mM NaCl, pH 7.5.

IC Diluent #10 - 50 mM HEPES, 0.1 mM EGTA, 0.1 mM EDTA, 120 mM NaCl, 0.5% NP-40 Alternative, pH 7.5. Store at 2 - 8° C for up to 3 months.

IC Diluent #11 - 10 mM HEPES, 0.1 mM EGTA, 0.1 mM EDTA, 0.5% BSA*, 1 mM DTT, pH 7.5. Prepare fresh just before use.

Note: *The presence of DTT is essential to measure phosphatase activity.*

Lysis Buffer #8 - 50 mM HEPES, 0.1 mM EGTA, 0.1 mM EDTA, 120 mM NaCl, 0.5% NP-40 Alternative (pH 7.5), 25 µg/mL Leupeptin, 25 µg/mL Pepstatin, 2 µg/mL Aprotinin, 1 mM PMSF. Prepare fresh just before use.

Note: *Lysis Buffer #8 consists of IC Diluent #10 plus 25 µg/mL Leupeptin, 25 µg/mL Pepstatin, 2 µg/mL Aprotinin, and 1 mM PMSF. Approximately 50 mL of IC Diluent #10 is required to run the assay on one 96 well plate.*

*The use of Bovine Serum Albumin (Sigma, Catalog # A7030) is recommended. All buffers containing BSA must be stored at 2 - 8° C.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Active SHP-2 Immunoprecipitation Beads (Part 841914) - 1 mL of rat anti-human antibody conjugated to agarose beads. Upon opening store at 2 - 8° C for up to 3 months.* **Do not freeze.**

Tyrosine Phosphatase Substrate I (Part 841915) - 13.0 mg/mL of DADEY(PO_3)LIPQQG when reconstituted with 60 µL of deionized water. After reconstitution, store at ≤ -20° C in a manual defrost freezer or at ≤ -70° C for up to 3 months.*

Phosphate Standard (Part 892809) - 1 mL of 1 M Phosphate (KH_2PO_4). A seven point curve using 2-fold serial dilutions and a high standard of 100 µM is recommended. Store at room temperature for up to 3 months.*

*Provided this is within the expiration date of the kit.

PREPARATION OF SAMPLES

Note: *Immediately before harvesting cells, prepare Lysis Buffer #8 and store on ice until use.*

Cell Lysates - Rinse cells two times with TBS, making sure to remove any remaining TBS after the second rinse. Solubilize cells at 1 x 10^7 cells/mL in Lysis Buffer #8 and allow samples to sit on ice for 15 minutes. Assay immediately or store at ≤ -70° C. Before use, centrifuge samples at 2000 x g for 5 minutes and transfer the supernate to a clean test tube. Sample protein concentration may be quantified using a total protein assay. If needed, further dilutions should be made in IC Diluent #10.

PREPARATION OF CONTROLS

The following controls may be included with samples being assayed.

	Control	1 mM Sodium Orthovanadate	Purpose
1	No Lysate	–	To determine non-enzymatic substrate hydrolysis and phosphate contamination.
2	Lysate	+	To determine phosphatase activity due to non-protein tyrosine phosphatases.

PRECAUTION

The Malachite Green Reagent A, Malachite Green Reagent B, and Phosphate Standard supplied with this kit are acidic solutions. Wear eye, hand, face, and clothing protection when using this material.

TECHNICAL HINTS AND LIMITATIONS

- This DuoSet IC activity assay should not be used beyond the expiration date on the kit label.
- Individual results may vary due to differences in technique, plasticware, and water sources.
- Use a fresh reagent reservoir and pipette tips for each step.
- This kit uses a highly sensitive phosphate detection method. Many soaps and dish detergents contain phosphate and will leave a residue that will increase the background absorbance of the assay. Containers should be rinsed extensively with distilled or deionized water before use.
- It is recommended that all standards and samples be assayed in duplicate or triplicate.
- Avoid microbial contamination of reagents and buffers. This may interfere with the sensitivity of the assay. Buffers containing protein should be made under aseptic conditions and stored at 2 - 8° C or be prepared fresh.

GENERAL ASSAY PROTOCOL

1. Mix IP beads by gentle vortexing and pipette 20 µL into a microcentrifuge tube for each sample.
2. Add 200 µL of IC Diluent #10 to each tube. Centrifuge briefly and aspirate off supernatant. There should be a packed pellet of about 10 µL. Repeat the wash step once more.
3. Thaw lysate and keep on ice until ready for use.
4. Dilute lysate with IC Diluent #10. The degree of dilution will vary with the cell line used. For initial experiments, several different dilutions should be tested. Add 100 µL of diluted lysate per reaction to the IP pelleted beads. For Control 1 (no lysate), add 100 µL of IC Diluent #10.
5. Shake the reactions at approximately 600 - 700 rpm at 2 - 8° C for 3 hours.

   Note: *It is critical that the IP beads remain suspended. Settled IP beads do not bind SHP-2 well.*
6. Centrifuge briefly and aspirate off the lysate.
7. Add 200 µL of IC Diluent #10. Vortex and centrifuge briefly. Aspirate off the supernatant.
8. Repeat the wash.
9. Wash once with 200 µL of IC Diluent #11.

   Note: *The presence of DTT in IC Diluent #11 is essential to measure phosphatase activity.*
10. Add 40 µL of IC Diluent #11 to all reactions. For Control 2 (non-specific activity), add 5 µL of 10 mM sodium orthovanadate.
11. Dilute the Tyrosine Phosphatase Substrate ten-fold with IC Diluent #11.
12. Add 10 µL of diluted Tyrosine Phosphatase Substrate I to each reaction. Mix by gentle tapping.
13. Shake the reactions at approximately 600 - 700 rpm at 37° C for 30 minutes. An orbital shaker inside an air incubator is recommended for this step.

    Note: *It is critical that the IP beads remain suspended. Settled IP beads have poor contact with the phosphatase substrate.*
14. During the 30 minute incubation in step 13, prepare the Phosphate Standard. A seven point standard curve using 2-fold serial dilutions and a high standard of 100 µM is recommended.

    Note: *The 1 M Phosphate Standard will precipitate in the presence of divalent cations such as calcium, magnesium or manganese. If divalent cations have been added to IC Diluent #11, dilute the Phosphate Standard to 10 mM with distilled or deionized water and further dilute with IC Diluent # 11. See Appendix for additional interfering substances.*

Prepare six 1:2 serial dilutions of the 100 μM Phosphate Standard in IC Diluent #11 (see Table 1).

Table 1: Dilution of standards for microplate assay.

Standard	Phosphate Conc.	nmol/well	Volume of IC Diluent #11	Volume of Standard
——	10 mM	——	990 μL	10 μL of 1 M Phosphate
1	100 μM	5	990 μL	10 μL of 10 mM Phosphate
2	50 μM	2.5	200 μL	200 μL of 100 μM Phosphate
3	25 μM	1.25	200 μL	200 μL of 50 μM Phosphate
4	12.5 μM	0.625	200 μL	200 μL of 25 μM Phosphate
5	6.25 μM	0.313	200 μL	200 μL of 12.5 μM Phosphate
6	3.13 μM	0.156	200 μL	200 μL of 6.25 μM Phosphate
7	1.56 μM	0.078	200 μL	200 μL of 3.13 μM Phosphate
Blank	——	0	200 μL	——

15. Use 50 μL of each standard in duplicate or triplicate to construct a standard curve.
16. Briefly centrifuge the samples and transfer 50 μL of the supernatant to wells of a microplate with the phosphate standard curve.
17. Add 10 μL of Malachite Green Reagent A to each sample, standard or blank. Mix thoroughly by tapping the side of the plate and incubate for 10 minutes at room temperature.
18. Add 10 μL of Malachite Green Reagent B to each well. Mix thoroughly by tapping the side of the plate and incubate the plate for 20 minutes at room temperature. This allows time for the yellow background color to bleach out.
19. Measure A_{620nm} on a microplate reader.

    Note: *The blue color will slowly fade over time. For best inter-assay consistency, read plates within 5 minutes of completing the incubation in step 18.*

CALCULATION OF RESULTS

Average the A_{620nm} readings of each standard or sample. Subtract the buffer blank from all standards and unknown samples. Plot nmol/well of Phosphate Standard vs. A_{620nm} (see Figure 1). Use computer software capable of simple linear regression or 4 parameter logistic curve fitting to create a standard curve. The phosphate content of unknown samples can then be calculated from the standard curve.

In the graph below, the Phosphate Standard was diluted in IC Diluent #11 and assayed in triplicate.

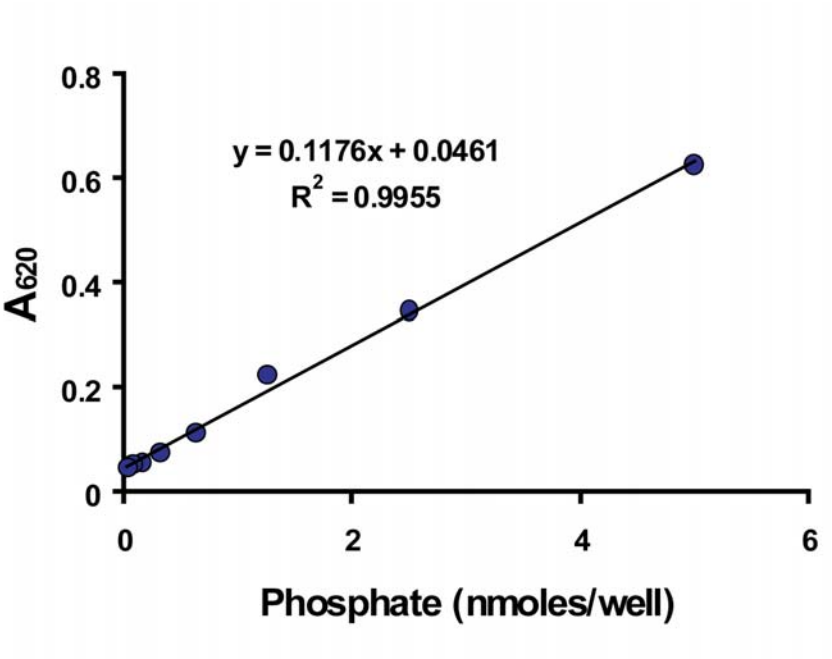


Figure 1: Assay for inorganic phosphate.

SPECIFICITY

This DuoSet IC activity assay is specific for SHP-2. Specificity was demonstrated by Western blot analysis of protein immunoprecipitated with the anti-SHP-2 IP beads supplied in the assay kit. One major band corresponding to SHP-2 was observed.

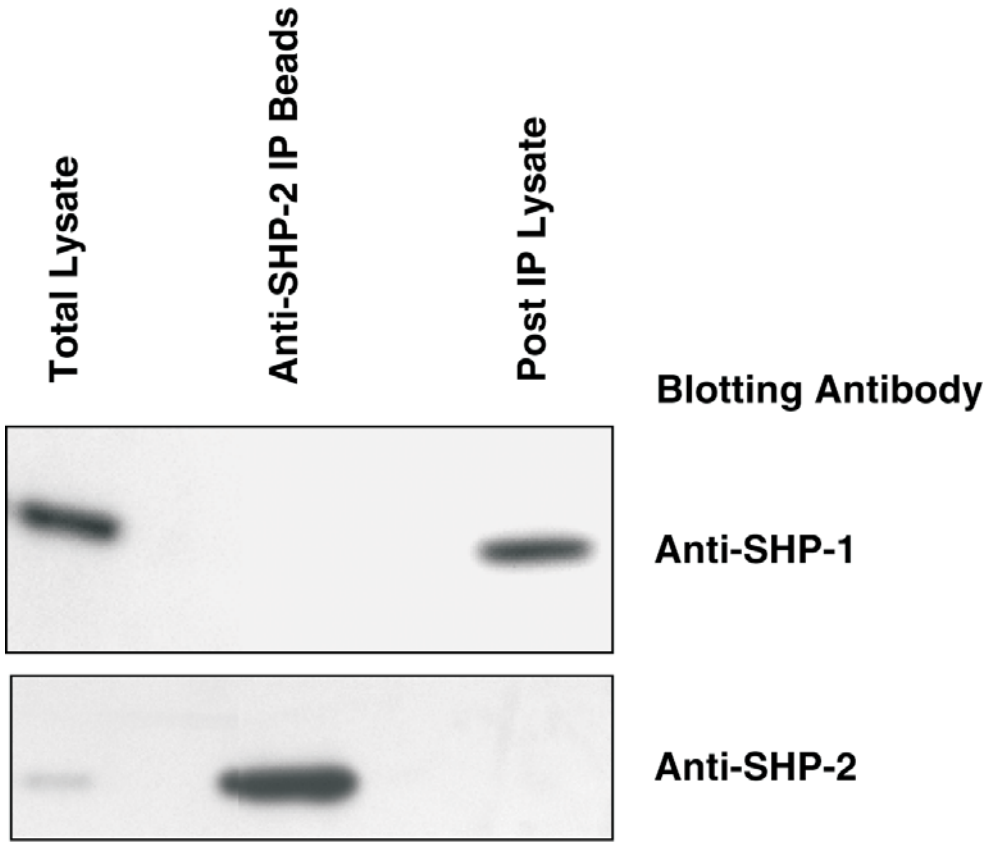


Figure 2: 20 µL of the anti-SHP-2 IP bead slurry was washed twice with IC Diluent #10, incubated with 100 µL of a Jurkat lysate at 2 - 8° C for 3 hours with shaking, and the IP beads pelleted by centrifugation. The post-immunoprecipitation lysate was collected and the IP beads were washed three times with IC Diluent #10. The samples were solublized in SDS-PAGE loading buffer and subjected to Western blotting using either anti-SHP-1 (R&D Systems, Catalog # AF1878) or anti-SHP-2 (R&D Systems, Catalog # AF1894) polyclonal antibodies. Both SHP-1 and SHP-2 were found in the original lysate but only SHP-2 was immunoprecipitated. SHP-1, which is 54% homologous to SHP-2, remained in the post-immunoprecipitation lysate.

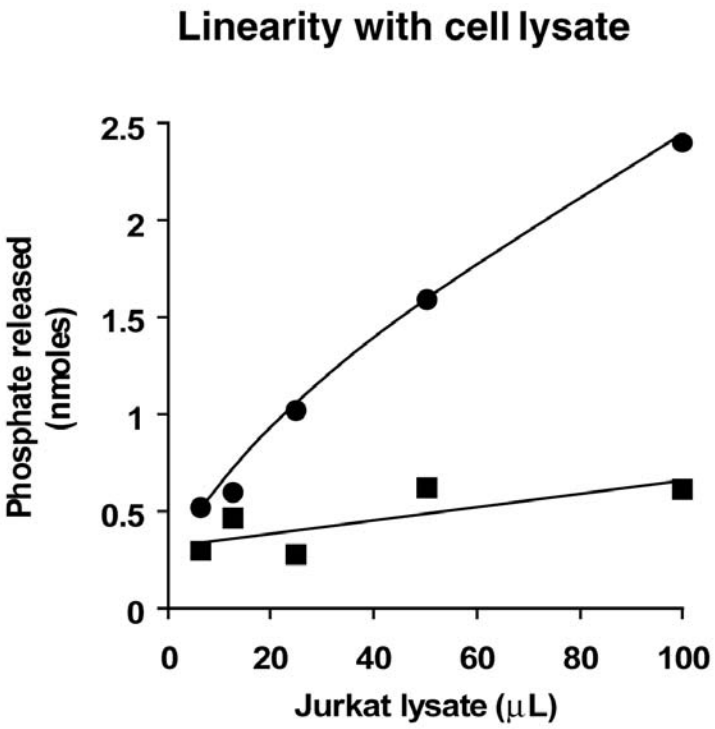


Figure 3: A Jurkat lysate prepared at 1 x 10^7 cells/mL was serially diluted prior to assay. To determine non-specific activity, 1 mM sodium orthovanadate was added to some of the samples. Circles indicate total phosphatase activity and squares indicate activity in the presence of 1 mM sodium orthovanadate.

Note: *If high activity samples are anticipated, a shorter reaction time or greater sample dilution is advised. When substrate dephosphorylation exceeds 2 nmoles per 30 minutes (20% of the total substrate), the slope of the curve decreases. A correction curve can also be generated by assaying dilutions of a cell lysate.*

QUANTIFICATION

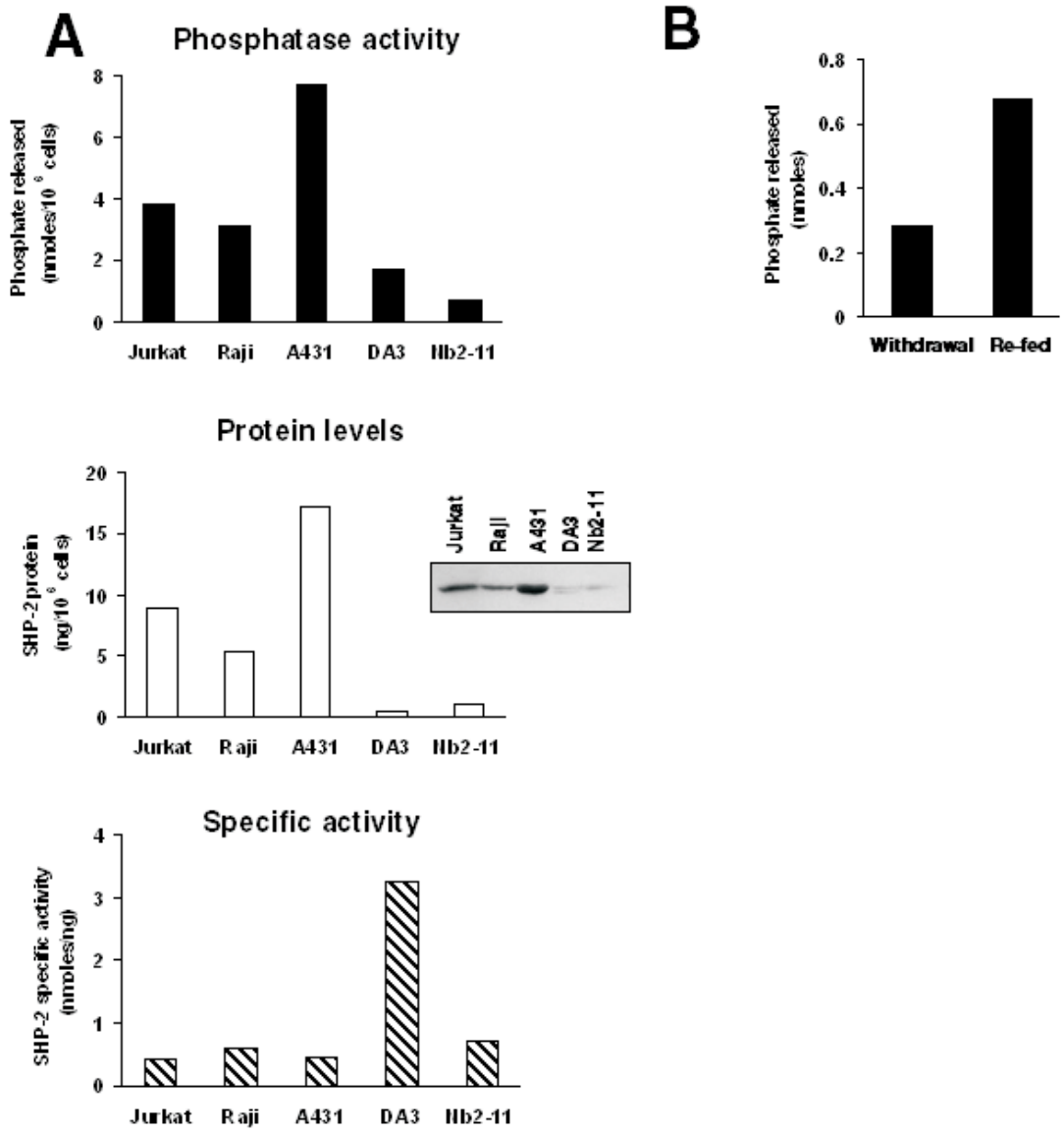


Figure 4:

A. In the top panel, SHP-2 phosphatase activity in lysates prepared from human Jurkat, Raji, and A431, mouse DA3 cells, and rat Nb2-11 cells was quantified using this assay. Non-specific activity was determined by adding 1 mM sodium orthovanadate to the assay buffer of some of the samples. Phosphatase activity is normalized to 1 x 10^6 cells. In the middle panel, the same lysates were also used to measure total SHP-2 protein with the Total SHP-2 Duoset IC ELISA (R&D Systems, Catalog # DYC1894) and by Western blot using an anti-SHP-2 antibody (R&D Systems, Catalog # AF1894) (inset). The bottom panel shows the specific activity of SHP-2, the ratio of the phosphatase activity to the SHP-2 protein levels. Note that despite markedly differing levels of SHP-2 protein and phosphatase activity, four of the five cell lines had roughly the same specific activity. Higher specific activity in DA3 cells is expected because they are grown in the presence of IL-3, which is known to increase SHP-2 phosphatase activity [Welham, M.J. *et al*. (1994) J. Biol. Chem. **269**:23764].

B. DA3 cells were cultured in serum- and IL-3-free medium for 5 hours (Withdrawal). Serum and IL-3 were then added to half of the cells for 40 minutes (Re-fed). Both groups of cells were lysed and SHP-2 phosphatase activity measured with this DuoSet IC activity assay. As expected, there was a two-fold increase in phosphatase activity in the re-fed cells.

APPENDIX

Substances tested for interference with phosphate determination

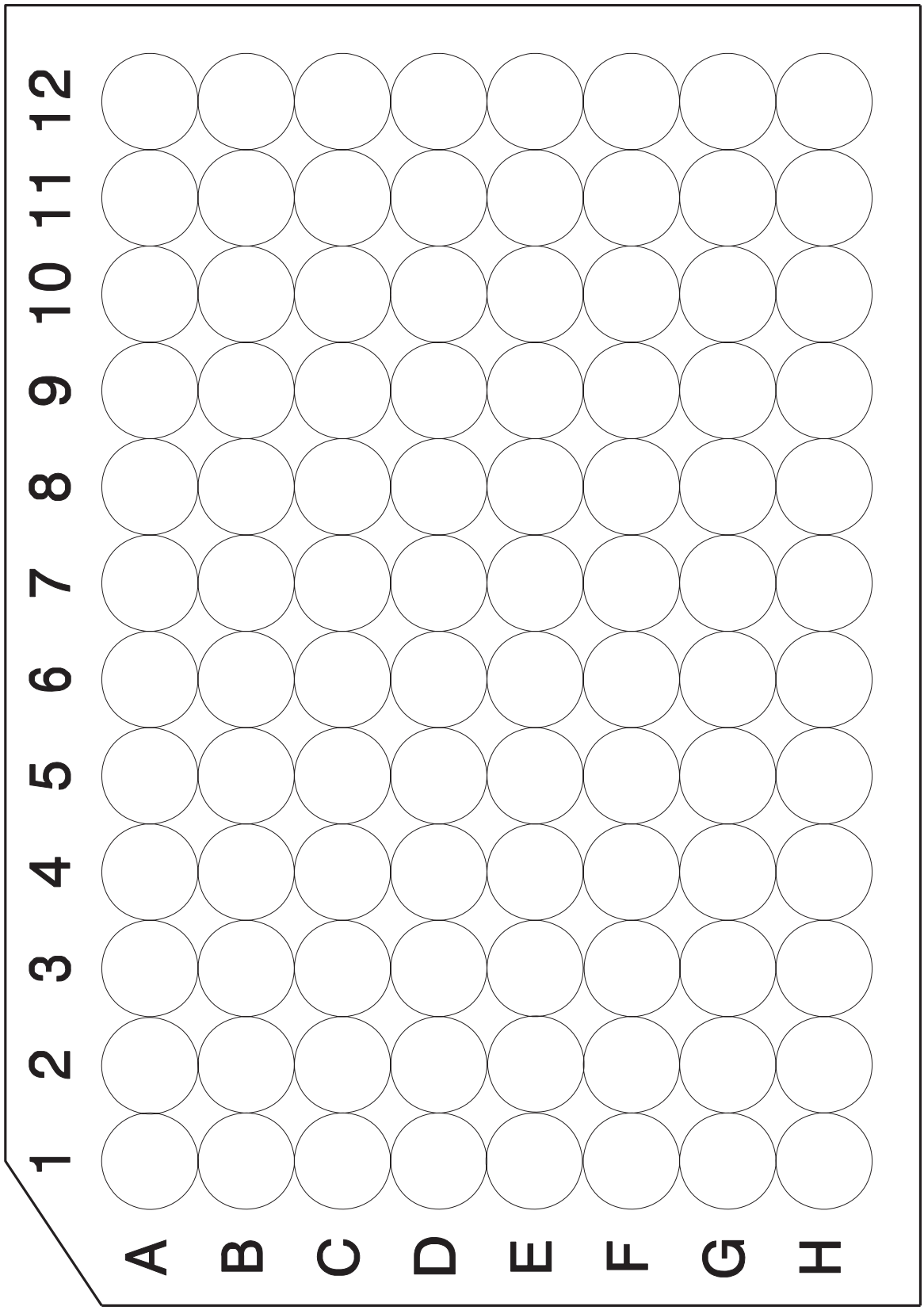
Buffers[1]	Compatible Concentrations	Adverse Effect at Higher Levels
Tris-HCl, pH 9.0	> 100 mM	—
HEPES, pH 7.5	> 100 mM	—
MOPS, pH 7.0	> 100 mM	—
Imidizole, pH 7.0	> 100 mM	—
MES, pH 5.5	> 100 mM	—
Detergents[2]		
Triton X-100	0.3%	Increased Background
Tween 20	0.1%	Reduces Sensitivity
NP-40	> 1%	—
CHAPS	> 1%	—
Deoxycholate	< 0.01%	Precipitates & Increases Background
SDS	< 0.01%	Increases Background
Common Reagents[2]		
Glycerol	5%	Reduces Sensitivity
DMSO	10%	Reduces Sensitivity
Ethanol	25%	Reduces Sensitivity
BSA	0.03 mg/mL	Reduces Sensitivity
EDTA	> 10 mM	—
Dithiothreitol	3 mM	Reduces Sensitivity
β-mercaptoethanol	> 10 mM	—
Na_3VO_4	1 mM	Reduces Sensitivity
NaF	> 10 mM	—
NaCl	> 100 mM	—
KCl	> 100 mM	—
$CaCl_2$	> 10 mM	—
Citric acid	10 mM	Reduces Sensitivity
ATP	100 μM	Increases Background Reduces Sensitivity

[1]Tested by microplate assay with or without 1 nmol of KH_2PO_4

[2]Tested by microplate assay in 25 mM Tris-HCl buffer (pH 7.5) with or without 1 nmol of KH_2PO_4

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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